The Ras effectors *NORE1A* and *RASSF1A* are frequently inactivated in pheochromocytoma and abdominal paraganglioma

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Abstract

NORE1A (*RASSF5*) and *RASSF1A* are newly described Ras effectors with tumour suppressor functions. Both molecules are frequently inactivated in various cancers. In this study, we aimed to explore the potential involvement of *NORE1A* and *RASSF1A* in pheochromocytoma and abdominal paraganglioma tumorigenesis. A panel of 54 primary tumours was analysed for *NORE1A* and *RASSF1A* mRNA expression by TaqMan quantitative RT-PCR. Furthermore, *NORE1A* and *RASSF1A* promoter methylation was assessed by combined bisulphite restriction endonuclease assay and methylation-sensitive Pyrosequencing respectively. The anti-tumorigenic role of *NORE1A* was functionally investigated in *Nore1A*-transfected PC12 rat pheochromocytoma cells by fluorescent inhibition of caspase activity and soft agar assays. Significantly suppressed *NORE1A* and *RASSF1A* mRNA levels were detected in primary tumours compared with normal adrenal medulla ($P < 0.001$). Methylation of the *NORE1A* promoter was not observed in primary tumours. On the other hand, 9% (5/54) of the primary tumours examined showed *RASSF1A* promoter methylation greater than 20% as detected by Pyrosequencing. Methylation of the *RASSF1A* promoter was significantly associated with malignant behaviour ($P < 0.05$). Transient expression of *Nore1a* resulted in enhanced apoptosis and impaired colony formation in soft agar. Our study provides evidence that *NORE1A* and *RASSF1A* are frequently suppressed in pheochromocytoma and abdominal paraganglioma. Silencing of *NORE1A* contributes to the transformed phenotype in these tumours.

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Introduction

The Ras family consists of small GTP-binding proteins with an established role in basic cellular functions related to growth, differentiation and apoptosis. Upon activation, Ras molecules can contribute to tumorigenesis or to cellular tumour defence, apparently diverse effects that are likely related to the downstream interacting Ras effector molecules (Frame & Balmain

2000, Feig & Buchsbaum 2002). Several recent reports suggest the involvement of Ras effector molecules in neoplasia by the loss of normal growth inhibitory effects (Feig & Buchsbaum 2002, Agathangelou *et al.* 2005).

NORE1A (*RASSF5*) is a member of the Ras association domain family (RalGDS/AF-6; Tommasi *et al.* 2002, Hesson *et al.* 2003), and the gene locus

is located in the chromosomal region 1q32.1. Data from experimental model systems suggest that NORE1A can act as a tumour suppressor by inducing apoptosis and delaying cell cycle progression in various cancer cell lines (Khokhlatchev *et al.* 2002, Vos *et al.* 2003, Aoyama *et al.* 2004). *NORE1A* is often silenced in various human neoplasias, and promoter methylation has been demonstrated as the main underlying mechanism (Tommasi *et al.* 2002, Chen *et al.* 2003, Hesson *et al.* 2003, Vos *et al.* 2003). So far, *NORE1A* gene mutations have not been identified in primary tumours or cancer cell lines (Tommasi *et al.* 2002, Chen *et al.* 2003, Hesson *et al.* 2003). Deletions in 1q are not frequent events in human cancer.

The closely related gene family member *RASSF1A* is located in 3p21.3, a frequently deleted region in human neoplasias (Lerman & Minna 2000). *RASSF1A* is a *bona fide* tumour suppressor (Burbee *et al.* 2001, Dreijerink *et al.* 2001, Kuzmin *et al.* 2002, Chow *et al.* 2004, Hesson *et al.* 2004, Tommasi *et al.* 2005). *RASSF1A* inactivation has been observed in different tumour types, the most frequent way of inactivation being promoter hypermethylation (Hesson *et al.* 2004, Agathangelou *et al.* 2005, Dammann *et al.* 2005). *RASSF1A* has been shown to heterodimerize with NORE1A (Ortiz-Vega *et al.* 2002) and mediate pro-apoptotic signals via recruitment of the pro-apoptotic mammalian Ste20 related kinase (MST1) (Khokhlatchev *et al.* 2002, Praskova *et al.* 2004), suggesting that NORE1A and *RASSF1A* are involved in the same pro-apoptotic pathway.

Pheochromocytomas and abdominal paragangliomas (the latter are here referred to as paraganglioma and in the literature often as extra-adrenal pheochromocytoma) are neuroendocrine tumours arising from neural crest-derived chromaffin cells. Pheochromocytomas are present in the adrenal medulla, whereas paragangliomas occur along the paravertebral and para-aortic sympathetic paraganglia. Pheochromocytomas frequently exhibit loss of *RASSF1A* expression by promoter methylation or deletion (Astuti *et al.* 2001, Dammann *et al.* 2005). To our knowledge, no study has investigated the involvement of *NORE1A* gene in these tumour entities. The current study was undertaken to examine the role of *NORE1A* and *RASSF1A* in pheochromocytoma and paraganglioma. Gene expression and promoter methylation status were assessed and evaluated in relation to various clinicopathological characteristics. Furthermore, we have enquired whether re-expression of *Nore1a* in PC12 pheochromocytoma cells can reverse the tumorigenic phenotype.

Materials and methods

Tumours and reference samples

Informed consent was obtained from all patients and the local ethics committee at the Karolinska University Hospital approved the study of the tissue material. Patients were operated at the Endocrine Surgical Unit at the Karolinska University Hospital Solna (Edström Elder *et al.* 2003). The study includes 56 tumours: 46 primary pheochromocytomas, 8 primary paragangliomas and 2 paraganglioma distant metastases. The clinical data are detailed for each case in Supplementary Table A which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>. Cases 2 and 19 as well as cases 14 and 27 are paired primary tumours and metastases. Seven tumours are from patients with a known familial form of the disease including multiple endocrine neoplasia type 2 (cases 11, 17 and 40), neurofibromatosis type 1 (cases 12, 23 and 41) and von Hippel Lindau disease (case 29). Before the analyses, representative sections were cut from all frozen tumour specimens and histopathologically evaluated to confirm the high proportion of tumour cells (>70%). Tumours were classified according to the criteria of the US Armed Forces Institute of Pathology, i.e. the diagnosis of malignancy required an extensive local invasion and/or distant metastasis (Lack 1997).

The following samples were used as normal controls for *NORE1A* and *RASSF1A* mRNA expression measurements: pooled human RNA from healthy adrenal glands (NA_{pool}) of 61 Caucasian subjects deceased of sudden death, purchased from Clontech Laboratories, Inc., Mountain View, CA, USA (reference number: 636528, ages 15–61), and adrenal medullary RNA (Norm 1–8) derived from eight Caucasian individuals (ages 50–76), who had no medical history of neoplastic disease and died due to cancer-unrelated causes (Clinomics Biosciences, Inc., Watervliet, NY, USA). For the methylation analyses, DNA derived from three histopathologically verified normal adrenal tissue specimens were used (Norm 9–11). Two of these patients were subsequently diagnosed with benign cystic lesions without hormonal abnormalities, and one was diagnosed with a small adrenocortical adenoma with surrounding normal adrenal tissue.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using RNeasy RNA extraction kit (Qiagen) and quantified by spectrophotometry. RNA integrity was confirmed by electrophoresis in 1% denaturing agarose gels, whereby the 18S and 28S bands were clearly demonstrated. Total

RNA (1.5 µg) was reverse transcribed in 100 µl reactions using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. qRT-PCR was performed using ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Gene-specific primers and probes available as TaqMan Gene Expression Assays (Applied Biosystems) were used, including assays Hs00417514_m1 for *NORE1A* and Hs00945257_m1 for *RASSF1A*. Sixty-five nanograms of each sample were amplified in a TaqMan 2X Universal Master Mix (final concentration 1×) under the following conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To select the most appropriate endogenous controls, we tested four different house-keeping genes across the samples: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β -actin (*ACTB*), β -2-microglobulin (*B2M*) and hypoxanthine guanine phosphoribosyltransferase 1 (*HPRT1*). Three of these showed relatively stable expression across the tumour samples and were selected for further analysis: *HPRT1* (Hs99999909_m1), *ACTB* (Hs99999903_m1) and *B2M* (Hs00187842_m1). All qRT-PCR experiments were performed in duplicate on two different occasions and included no-template controls. Serial dilutions of NA_{pool} cDNA were amplified in parallel to establish a standard curve for relative quantification.

The raw data were analysed using Sequence Detection System (SDS 1.9.1, Applied Biosystems) and quantified in relation to the generated standard curves. The expression values of each target gene were then normalized to the geometrical average of the three housekeeping genes (*HPRT1*, *ACTB* and *B2M*) and subsequently to the reference NA_{pool} , which was assigned a nominal value of 1.

DNA extraction and bisulphite treatment

High molecular weight DNA was extracted using standard procedures with proteinase K digestion, phenol–chloroform extraction and ethanol precipitation. All bisulphite treatment was done using EZ DNA Methylation Kit (Zymo Research Corporation, Orange, CA, USA), following the manufacturer's instructions.

Combined bisulphite restriction endonuclease assay (COBRA)

The *NORE1A* promoter was amplified from bisulphite-treated DNA of tumours and normal controls (Norm 9–10) by nested PCR as described (Chen *et al.* 2003). PCR products were purified using Microcon YM-100 (Millipore Corporation, Billerica, MA, USA) and

cleaved by *TaqI* (Invitrogen Life Technologies, Inc.). Genomic DNA from MCF-7 cells was used as a positive control for the methylated state (Chen *et al.* 2003), while human leukocyte DNA from healthy individuals served as an unmethylated assay control. DNA from Norm 9–10 was used as a healthy reference. Results were confirmed by repeated analyses on two different occasions.

Quantification of RASSF1A methylation by Pyrosequencing

Bisulphite-treated DNA from tumours and normal controls (norm 11) were used for PCR amplifications. Pyrosequencing was performed using the PSQHS96 system (Biotage AB, Uppsala, Sweden) including PyroGold reagents. Primers were designed by Biotage AB and are available at the PyroMark Assay Database (Biotage AB). The primers amplify a stretch of the *RASSF1A* exon 1 (Ensemble ID: ENST00000266020). The outer primers target CpG-free regions within this stretch. PCR was carried out using 1.5 µl bisulphite-treated DNA under the following conditions: 95 °C for 15 min, 45× (95 °C for 20 s, 55 °C for 20 s, 72 °C for 20 s), 72 °C for 10 min, 4 °C. Intactness of the PCR product was assessed by electrophoresis on 3% agarose gel and subsequent ethidium bromide staining. Pyrosequencing was carried out at Biotage AB. The Pyrosequencing primers target a 24-nt segment, which lies within the previously amplified stretch of *RASSF1A* exon1 and contains five CpGs (CCGCCCGGCCCGCGCTTGCTAGCG). This segment was chosen to cover the sites analysed for *RASSF1A* promoter methylation in previous reports (Honorio *et al.* 2003).

Transfection of PC12 cells with Nore1a constructs

Generation, sequencing and characterization of rat *NORE1A* (*Nore1a*) cDNA constructs have previously been published (Vos *et al.* 2003). Briefly, *Nore1a* cDNA was cloned into pcDNAF, a modified version of pcDNA3 (Invitrogen Life Technologies, Inc.), which attaches a FLAG tag to the insert (pcDNAF~Nore1a). To generate the green fluorescent protein (GFP) ~Nore1a construct rat, *Nore1a* cDNA was inserted into a Clontech's GFP vector (Clontech Laboratories, Inc.). The correct sizes of the inserts were confirmed by the restriction endonuclease cleavage followed by agarose gel electrophoresis. PC12 rat pheochromocytoma cells were grown using standard reagents and procedures. Transient transfections were carried out with GFP~Nore1a and pcDNAF~Nore1a using LipofectAmine 2000

(Invitrogen Life Technologies, Inc.), according to the manufacturer's instructions. PC12 cells were transiently transfected with pcDNAF~Nore1a and GFP at a molar ratio of 10:1 or GFP~Nore1a. Medium was changed after 12 h. Successful transfection was confirmed after 24 h by fluorescent microscopy and western blot analyses (data not shown).

Apoptosis assay

To determine the fraction of apoptotic cells after transfection, Sulphorhodamine Apoptosis Detection Kit Caspase Assay was used (Immunochemistry Technologies LLC, Bloomington, MN, USA). Briefly, cells were harvested 24 h after transfection, resuspended in the culture medium and analysed by fluorescent inhibition of caspase activity (FLICA), according to the manufacturer's instructions. Cells were analysed with a FACScalibur (BD Biosciences, San Jose, CA, USA) flowcytometer. The green fluorescent cell population, i.e. transfected cells, was assessed for red fluorescence (FLICA), indicating binding to active caspases.

Soft agar assay

Twenty-four hours after transfection, cells were harvested, fluorescence activated cell sorting (FACS) sorted for green fluorescence and replated for an additional 36 h to allow recovery from cellular shock caused by FACS. Cells were subsequently harvested, counted and seeded at a density of 1250 cells per well in 0.3% soft agar, which was overlaid onto 0.6% bottom agar (Agarose type VII, Sigma-Aldrich). Cells were fed every 4–5 days. After 11 days, cells were stained with MTT (Roche AB) overnight and colonies were counted.

Statistical analyses

All analyses were performed in STATISTICA data analysis software system version 7 (Statsoft, Inc., Tulsa, OK, USA), and for all tests, P -values ≤ 0.05 were considered significant. Statistical analyses comprised data only from the 54 primary tumour samples, while the two metastases were excluded. Expression values for *NORE1A* and *RASSF1A* were compared between groups of samples with different clinical and histopathological characteristics, as well as between tumour samples and normal controls. Mann–Whitney test was used to assess differences in target gene expression between tumours versus normal controls, females versus males, paragangliomas versus pheochromocytomas and benign versus malignant disease. Correlation between tumour size and target gene expression, as well as between age at presentation

and target gene expression, was examined using Spearman rank-order correlations. To assess correlations between the degrees of methylation at each of the five CpG sites within the *RASSF1A* promoter tested by Pyrosequencing, and the different clinical and histopathological tumour features, we used Mann–Whitney test and Spearman rank-order correlations. Furthermore, Spearman rank-order correlations and Kruskal–Wallis analysis were utilized to assess the relationship between *RASSF1A* promoter methylation and *RASSF1A* mRNA expression.

Results

Reduced *NORE1A* and *RASSF1A* mRNA expression in pheochromocytoma and paraganglioma

Significantly reduced *NORE1A* mRNA levels were detected in primary tumours when compared with normal adreno-medullary control samples ($P=0.00001$; Mann–Whitney test; Fig. 1A). No significant correlations were detected between *NORE1A* mRNA levels and the various clinicopathological characteristics.

RASSF1A was substantially under-expressed in primary tumours when compared with normal adreno-medullary control samples ($P=0.000018$; Mann–Whitney test; Fig. 1B). There was no significant correlation between *RASSF1A* mRNA levels and benign versus malignant characteristics, tumour type, sex or tumour size (Mann–Whitney test and Spearman

Figure 1 (A) Detection of reduced *NORE1A* mRNA expression in primary pheochromocytomas and paragangliomas. Nominal value of 1 corresponds to the expression level measured in a pool of total adrenal gland extracts from 61 individuals. The box plot demonstrates that the overall *NORE1A* mRNA expression in tumours is significantly lower than that in the eight normal adreno-medullary reference samples. (B) Detection of reduced *RASSF1A* mRNA expression in primary pheochromocytomas and paragangliomas. Values are normalized to the expression level measured in a pool of total adrenal gland extracts from 61 individuals. The box plot demonstrates that the overall *RASSF1A* mRNA expression in tumours is significantly lower than that in the eight normal adreno-medullary reference samples.

Figure 2 COBRA analysis of the *NORE1A* promoter. PCR product from bisulphite-treated DNA was cleaved with *TaqI*. The analysed 325 bp of the *NORE1A* promoter contains 35 CpG sequences. If this fragment is methylated, the two *TaqI* sites will be cleaved, resulting in the bands of 202, 123 and 10 bp. Top panel: MCF-7 (M) cell line was used as a positive control for the methylated status, which clearly demonstrates partial restriction cleavage indicating partial methylation. U, human lymphocyte DNA was used as a negative control for the unmethylated status; L, lymphocyte DNA without bisulphite treatment; H₂O, water control. Four representative tumours are shown to the right. Only tumour 27, a distant metastasis, showed modest partial methylation as demonstrated by weak partial cleavage. Tumours 1, 2 and 29 are without methylation in the *NORE1A* promoter. A 123 bp ladder is used as a size marker.

rank-order correlations). However, a tendency towards lower *RASSF1A* expression was observed in tumours from patients with a younger age at diagnosis ($P < 0.05$; $R = 0.29$; Spearman rank-order correlations).

***NORE1A* promoter methylation is a rare event in pheochromocytoma and paraganglioma**

Methylation of the *NORE1A* promoter was not detected in the normal adrenal reference samples or in the primary tumours. Weak partial methylation of

the *NORE1A* promoter was observed in one tumour (sample no. 27), which was a metastasis obtained from a patient with malignant paraganglioma (Fig. 2).

Quantification of *RASSF1A* methylation by pyrosequencing

The five CpG sites were analysed quantitatively by methylation-sensitive Pyrosequencing (Fig. 3; Table 1). Normal reference adrenal DNA exhibited a low level of methylation (below 10%) at the CpG sites assessed. Significantly higher degree of methylation was observed in primary malignant tumours compared with benign ones ($P < 0.05$; Mann–Whitney test). For all five CpG sites, tumours were categorized into groups according to the methylation value, i.e. low methylation, intermediate and high methylation. Groups were then compared with regard to *RASSF1A* mRNA expression. No difference was seen between the groups with regard to *RASSF1A* expression (Kruskal–Wallis analysis). No significant correlation was seen between the methylation degree at the CpG sites tested and the *RASSF1A* expression (Spearman rank-order correlation). Both metastases as well as their original primary tumours showed high levels of *RASSF1A* promoter CpG methylation.

Reduced colony formation after *Nore1A* transfection in PC12 cells

More than two- and ninefold reduction in colony formation was observed by GFP~*Nore1a*- and pCDNAF~*Nore1a*-transfected PC12 cells respectively when compared with negative controls (GFP-transfected cells; $P < 0.05$; Fig. 4).

Figure 3 Pyrogram from quantification of methylation by Pyrosequencing of the *RASSF1A* promoter. During the Pyrosequencing reaction, a 'C' is incorporated if the template CpG is methylated, while a 'T' is incorporated if the template CpG is unmethylated. In the resulting pyrogram, the proportion of C:T reflects the degree of methylation at the particular CpG sites assessed.

Table 1 Results from the studies by qRT-PCR and promoter methylation analyses

Case no.	RASSF1A mRNA	NORE1A mRNA	RASSF1A methylation by pyrosequencing						NORE1A COBRA
			Pos 1	Pos 2	Pos 3	Pos 4	Pos 5	Average	
1	0.205	0.305	1.3	1.7	1.5	1.1	1.4	1.4	Unmeth
2	0.21	0.32	57.9	60.6	62.2	53.5	52.6	57.36	Unmeth
3	1.44	0.14	2.6	2.7	12	10.6	16.7	8.92	Unmeth
4	0.245	0.01	0	2.4	0	1.1	0	0.7	Unmeth
5	0.755	0.08	0	1	1.2	0	0	0.44	Unmeth
6	0.815	0.15	10.5	7.1	5.4	7.3	12.6	8.58	Unmeth
7	0.225	0.14	3	2.1	2.1	2.8	3.3	2.66	Unmeth
8	0.22	0.455	3.1	6.6	4.3	11.6	5.4	6.2	Unmeth
9	0.155	0.03	24.5	5	4.1	5.3	6.7	9.12	Unmeth
10	0.74	0.695	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Unmeth
11	0.4	0.4	19.2	3.9	2.8	3.2	24.6	10.74	Unmeth
12	0.26	0.15	1.7	1.7	1.6	1.4	2.3	1.74	Unmeth
13	0.49	0.13	5.3	3.5	3.4	5	6.4	4.72	Unmeth
14	0.265	0.14	66.5	68.4	40.5	57.6	58.8	58.36	Unmeth
15	0.16	0.79	2.3	3.9	12.6	11.5	3.5	6.76	Unmeth
16	0.72	0.095	1	1.2	1.2	0.9	1	1.06	Unmeth
17	0.115	0.01	1.9	2	1.6	1.9	2.1	1.9	Unmeth
18	0.33	0.45	5.2	2.6	2.6	3.4	29.1	8.58	Unmeth
19	0.165	1.77	50	54	53.2	47.2	45.3	49.94	Unmeth
20	0.32	0.02	6.1	29.4	2.7	2	2.3	8.5	Unmeth
21	0.225	0.105	1	1.3	1.5	1.1	1.3	1.24	Unmeth
22	0.28	0.185	19.2	3.8	3.1	3.2	19.7	9.8	Unmeth
23	0.275	0.36	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Unmeth
24	0.215	0.125	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Unmeth
25	0.27	0.11	1	1.2	1.4	0.9	1.1	1.12	Unmeth
26	0.355	0.155	70.2	71.1	72.2	63	61.1	67.52	Unmeth
27	0.255	0.045	69.5	72.5	72.2	64	62	68.04	Part meth
28	0.375	0.265	1.7	2.6	5	2.4	2.8	2.9	Unmeth
29	0.495	0.345	0	1.2	1.3	0.8	0.8	0.82	Unmeth
30	0.695	0.155	24.6	2.9	2.2	2.2	20.5	10.48	Unmeth
31	0.23	0.075	3.4	3	3.9	25.7	9.8	9.16	Unmeth
32	0.22	0.25	1.2	1.4	2	2.7	1.9	1.84	Unmeth
33	0.285	0.195	36.1	7.7	23.4	10.5	16.6	18.86	Unmeth
34	0.37	0.025	28	3.7	3.2	1.9	2.7	7.9	Unmeth
35	0.25	0.125	0.8	1.3	1.4	0.8	0.8	1.02	Unmeth
36	0.265	0.065	1.5	1.7	1.6	1.3	1.3	1.48	Unmeth
37	0.13	0.925	0	0	10.2	0	17.4	5.52	Unmeth
38	0.1	0.025	5.7	5.7	10.9	6.9	10.7	7.98	Unmeth
39	0.16	0.08	7.8	5	5.5	4.4	10.8	6.7	Unmeth
40	0.15	0.23	1	1.5	1.6	1	1	1.22	Unmeth
41	0.21	0.285	0	0	0	0	0	0	Unmeth
42	0.045	0.015	23.4	17.4	12.2	23.3	28	20.86	Unmeth
43	0.3	0.4	0	1.2	1.2	0.6	0.7	0.74	Unmeth
44	0.135	0.015	4.1	3.3	2.9	3.8	11	5.02	Unmeth
45	0.4	0.125	1.5	1.6	1.3	1.1	0.8	1.26	Unmeth
46	0.21	0.14	7.4	31.1	6.9	5.3	10.3	12.2	Unmeth
47	0.5	0.08	6.7	23.3	29.4	9.1	28	19.3	Unmeth
48	0.135	0.065	7.2	7.8	5.2	10	29.7	11.98	Unmeth
49	0.31	0.285	0.9	1.7	1.3	0.9	1.1	1.18	Unmeth
50	0.275	0.155	5.3	2	1.9	1.8	4.1	3.02	Unmeth
51	0.515	0.455	45.7	41.4	9.9	21.3	24.4	28.54	Unmeth
52	0.325	0.18	1.7	1.9	1.8	1.7	2	1.82	Unmeth
53	0.295	0.025	0	0	1.9	0	0	0.38	Unmeth
54	0.48	0.105	20.8	5.2	3.8	3.7	2.9	7.28	Unmeth
55	0.34	0.13	9.7	28.3	7.8	18.1	25.3	17.84	Unmeth
56	0.225	0.035	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Unmeth

n.d., not determined; Part meth, partially methylated; Unmeth, unmethylated; Pos 1–5, CpG positions.

Figure 4 Top: reduced anchorage-independent growth quantified by the number of colonies of PC12 cells after transfection with GFP~Nore1 or pcDNAF~Nore1a when compared with only GFP-transfected cells. The results show a composite value from three measurements. Bottom: representative images of soft agar gel assay with corresponding constructs (GFP, GFP~Nore1a and pcDNAF~Nore1a).

Increased apoptotic rate in PC12 cells transfected with Nore1a

Transfection of PC12 cells with pcDNAF~Nore1a or GFP~Nore1a was associated with a 1.5- and 2-fold increase in apoptotic rate respectively when compared with GFP-transfected cells used as negative controls (Fig. 5).

Discussion

NORE1A is a downstream Ras effector that mediates inhibitory effects with regard to cellular growth and survival. NORE1A can induce apoptosis and cell cycle progression delay in cancer cells (Vos *et al.* 2003, Aoyama *et al.* 2004). NORE1A expression is frequently down-regulated or lost in various human cancer cell lines and tumours. Our present observation of markedly decreased *NORE1A* mRNA expression in pheochromocytoma and paraganglioma is in agreement with previous reports demonstrating suppressed *NORE1A* expression in other types of neoplasias (Tommasi *et al.* 2002, Chen *et al.* 2003, Hesson *et al.* 2003, Vos *et al.* 2003, Aoyama *et al.* 2004).

Several different mechanisms could potentially lead to gene silencing, including structural mutations, deletions, epigenetic modifications and other regulatory mechanisms at the transcriptional

or post-transcriptional level. Epigenetic inactivation by promoter methylation has been implicated as a common mechanism underlying *NORE1A* silencing (Tommasi *et al.* 2002, Chen *et al.* 2003, Vos *et al.* 2003, Hesson *et al.* 2004). In view of these observations, and our present finding of frequent suppression of *NORE1A* mRNA expression, we have assessed *NORE1A* promoter methylation using COBRA assay (Chen *et al.* 2003). In contrast to results from other tumour types, we have found that *NORE1A* promoter methylation is a rare event in pheochromocytomas and paragangliomas. Only one tumour, a distant metastasis from a malignant pheochromocytoma, showed slight

Figure 5 FLICA apoptosis assay of pcDNAF~Nore1a- and GFP~Nore1a-transfected PC12 cells. Green fluorescent (transfected) cells were gated and further analysed for red fluorescent (FLICA) signal by FACS. Results show that transfection by pcDNAF~Nore1a and GFP~Nore1a resulted in a 1.5- and 2-fold increase in apoptotic rate respectively when compared with GFP-transfected cells used as negative controls.

partial methylation. So far, no inactivating mutations in the *NORE1* gene have been reported in tumours or cancer cell lines (Tommasi et al. 2002, Chen et al. 2003, Hesson et al. 2003, Vos et al. 2003, Hesson et al. 2004). The *NORE1* gene is mapped to 1q32.1-2, a region that is only rarely affected by somatic deletions in human cancer. Our previous comparative genomic hybridisation (CGH) results on pheochromocytomas and paragangliomas showed no indication of major DNA loss on 1q (Edström et al. 2000). Therefore, it does not appear likely that the observed *NORE1A* transcriptional silencing is due to allelic loss or mutation. This however does not exclude the possibility of smaller allelic losses in the region beyond the resolution of CGH.

Restoration of NORE1A expression in cell lines lacking endogenous expression is associated with reduced cell growth, delayed cell cycle progression, enhanced apoptosis and reduced anchorage-independent growth (Tommasi et al. 2002, Chen et al. 2003, Hesson et al. 2003, Vos et al. 2003, Aoyama et al. 2004). To enquire whether impaired NORE1A expression affects the propensity of pheochromocytoma cells to undergo apoptosis, we have assessed apoptotic rate in *Nore1a*-transfected PC12 cells using a fluorescent pan caspase detection system. Increased apoptotic rate was detected amongst transfected cells, indicating that loss of *NORE1A* expression in pheochromocytomas may impair the cells' ability to perish via programmed cell death, an observation that was also suggested by Vos et al. (2003).

The capacity of tumour cells to grow in an anchorage-independent fashion is a good indicator of their *in vivo* tumorigenic potential (Cifone 1982). We have investigated the impact of *Nore1a* on the ability of PC12 cells to proliferate in soft agar. In agreement with other reports, we have observed substantially decreased colony numbers after *Nore1a* transfection, indicating that impairment of *Nore1a* expression may be an important contributing factor to the neoplastic growth of pheochromocytoma cells (Fig. 4; Vos et al. 2003).

The *RASSF1A* promoter is one of the most commonly hypermethylated tumour suppressor gene promoters in human cancer and its methylation is associated with the loss of *RASSF1A* gene expression (Dammann et al. 2000, Astuti et al. 2001, Dammann et al. 2001, Murray et al. 2004). Astuti et al. observed 22% (5/23) of *RASSF1A* promoter methylation in sporadic pheochromocytomas (Astuti et al. 2001). In a recent report, Damman et al. detected 48% methylation (12/25) in a series of familial and sporadic pheochromocytomas (Dammann et al. 2005). However, both reports utilized non-quantitative methods, COBRA and methylation specific polymerase chain reaction (MSP) respectively, which could not quantitatively assess the proportion of

methyated to unmethyated CpGs at each of the CpG sites analysed. This may be particularly important considering that MSP can be sensitive to as little as 0.1% of methylated alleles (Herman et al. 1996), thus classifying a sample as methylated on the basis of a minor proportion of methylated target, which may not reflect the overall scenario within the tumour sample studied. In the study by Dammann et al. (2005) the cases positive for *RASSF1A* methylation mainly exhibited partial methylation, i.e. PCR product was detected with both methylated and unmethylated allele-specific primers. Similarly, Astuti et al. (2001) reported only partial digestion in their *RASSF1A* COBRA analyses in pheochromocytomas, and when DNA from *RASSF1A* methylated tumours was bisulphite treated and then sequenced, both thoroughly methylated and unmethylated clones were detected. These observations suggest that partial methylation may be attributed to the presence of non-neoplastic stromal cells or heterogeneity within the tumour cell population with regard to *RASSF1A* promoter methylation.

In view of these previous findings, we have proceeded to assess the methylation status of the *RASSF1A* promoter in a quantitative fashion using methylation-sensitive Pyrosequencing. This technique allows for the relative quantification of methylated CpGs in relation to unmethylated CpGs for each of the CpG sites tested. We have found that 9% (5/54) of the primary tumours examined showed *RASSF1A* methylation greater than 20% (as an average of methylation values of the five examined CpG sites). Three of these five tumours were primary malignant paragangliomas, one was a primary malignant pheochromocytoma and one was a benign pheochromocytoma (Table 1 and Supplementary Table A, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). Overall, malignant tumours showed significantly higher levels of methylation compared with benign ones ($P < 0.05$). This finding is consistent with other reports showing correlation between the prevalence of *RASSF1A* promoter hypermethylation and adverse tumour characteristics (Byun et al. 2001, Kuroki et al. 2003, Hesson et al. 2004, Kim et al. 2004). In the two cases of paired primary tumour and metastasis, both the primary lesions and the corresponding metastases showed high levels of methylation. This might suggest that methylation of the *RASSF1A* promoter is a relatively early event; however, one would need to study an extended number of paired primary tumours and metastases to ascertain this concept.

Pheochromocytomas and paragangliomas in this study displayed significantly suppressed *RASSF1A* gene expression levels compared with that measured

in non-neoplastic adrenal medulla. All samples that showed at least 20% of methylated alleles for *RASSF1A* promoter had greatly reduced expression. Although promoter hypermethylation is the predominant way of *RASSF1A* expressional suppression, one may consider other contributing mechanisms of tumour suppressor gene inactivation. Mutation of *RASSF1A* is rare, although not unprecedented in human cancer (Burbee *et al.* 2001, Dreijerink *et al.* 2001, Lo *et al.* 2001). However, Astuti *et al.* (2001) did not detect *RASSF1A* mutation in pheochromocytomas. On the other hand, loss of heterozygosity (LOH) at the 3p21.3 resident site where *RASSF1A* is located occurs frequently (38.5%) in pheochromocytomas (Astuti *et al.* 2001). Our findings suggest that methylation is not likely to fully account for the thoroughly suppressed *RASSF1A* mRNA levels in these tumours. It is thus highly possible that other mechanisms such as LOH or perturbed transcriptional regulation are significant contributing factors to *RASSF1A* inactivation in these tumours.

In conclusion, the present study demonstrates that pheochromocytomas and abdominal paragangliomas display markedly suppressed gene expression of two prominent members of the Ras association domain family, *NORE1A* and *RASSF1A*, both of which have been widely implicated in the defence against tumorigenic growth. The lack of correlation between the *NORE1A/RASSF1A* mRNA expression levels and the various clinicopathological features indicates a more general role for these molecules in pheochromocytoma/paraganglioma tumorigenesis that is not confined to a particular subgroup of patients. *RASSF1A* but not *NORE1A* promoter methylation is a frequent event in these tumours, the previous also showing a good correlation with malignant behaviour. Furthermore, we provide functional evidence that reconstitution of Nore1a expression in PC12 rat pheochromocytoma cells reduces the tumorigenic phenotype. This study is the first report investigating *NORE1A* in pheochromocytomas and paragangliomas, as well as revealing an association between the degree of *RASSF1A* promoter methylation and malignant behaviour in these tumours.

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